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Method of detaching microorganisms from, or of inhibiting microbial attachment to, animal or poultry carcasses or seafood or parts thereof

Reference to Related Application

This application claims the benefit of U.S. Provisional Application No. 60/196,092, filed 11 April 2000, which is incorporated herein by reference in its entirety.

Background Of The Invention

The present invention relates to a method of detaching microorganisms (e.g., bacteria) from, or of inhibiting microbial attachment to, animal or poultry carcasses or seafood or parts thereof, wherein the method involves contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of the following: (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from, or inhibit microbial (e.g., bacterial) attachment to, animal or poultry carcasses or seafood or parts thereof.

Food borne disease in the U.S. is estimated to occur between 6.5 to 33 million cases per year (Council for Agricultural Science and Technology (CAST), 1994, *Food borne Pathogens: Risks and Consequences*, Task Force Report No. 122). Bean et al. (Center for Disease Control (CDC) (1996), Surveillance for Foodborne-Disease Outbreaks-United States, 1988-1992, Center for Disease Control (CDC), Morbidity and Mortality Weekly Report, 45: 1-65) also reported that bacterial pathogens caused 79% of outbreaks and 90% of cases when the etiology was determined in food borne disease outbreaks in the U.S. from 1988-1992. *E. coli* O157:H7 outbreaks have caused several fatalities, particularly in children. Recently, Mead et al (Food-related illness and death in the United States, Emerg. Infect. Dis., 5: 607-625 (1999)) estimated that in the U.S. there are about 1.3 million cases of illness due to non-typhoidal salmonella (9.7% of total food borne pathogen diseases); 15,600 cases of hospitalization (25% of food borne pathogens); and 553 deaths (30.6% of food borne pathogens); these cases are 9.7%,

25% and 30.6% of the total food borne illnesses, hospitalizations and deaths, respectively. The estimates for *E. coli* O157:H7 illness, hospitalization and deaths are 62,458, 1,843 and 52 cases; their respective percent of the total food borne illnesses, hospitalizations and deaths are 0.5%, 3% and 2.9%, respectively. Productivity losses and medical costs were estimated to be in the range of $6.5 to $34.5 billion per year (CAST, 1994). Recent recalls of contaminated beef resulted in millions of dollars in losses for the food industry. Therefore, any new approach to reducing or eliminating pathogen contamination in foods would decrease food borne illness and medical costs and provide savings to the food industry and American taxpayers.

Summary Of The Invention

A method of detaching microorganisms (e.g., bacteria) from, or of inhibiting microbial attachment to, animal or poultry carcasses or seafood or parts thereof, wherein the method involves contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of the following: (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from, or inhibit microbial attachment to, animal or poultry carcasses or seafood or parts thereof.

Brief Description Of The Drawings

Figure 1 shows Beef Slaughter Plant Application;

Figure 2 shows Poultry (Chicken) Slaughter Plant Application;

Figure 3 shows Swine Slaughter Plant Application in Clean Room;

Figure 4 shows Detachment for Sampling for Laboratory Analysis;

Figure 5 shows Detachment for *In Situ* Sampling in Slaughter Plants;

Figure 6 shows structures of heparin sulfate and carrageenans;

Figure 7 shows photos of chicken skin inoculated with *Salmonella*;
Figure 8 shows photos of pork inoculated with *Salmonella*.

Figure 9 shows photos of detached coliform and *E. coli* from the surface of chicken legs.

Detailed Description Of The Invention

One aspect of the present invention involves a method of detaching microorganisms (e.g., bacteria) from, or of inhibiting (blocking) microbial attachment to, animal or poultry carcasses or seafood or parts thereof. The method involves contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of the following: (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween (e.g., Tween 80) and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from, or inhibit microbial attachment to, animal or poultry carcasses or seafood or parts thereof; these compounds may be dissolved or suspended in phosphate buffer. This method may be also used in detecting and quantifying microorganisms (e.g., bacteria) on carcasses or parts thereof where the microorganisms must first be detached from the carcasses or parts thereof. Other Tweens known in the art may be substituted for Tween 80.

The term "animal" herein includes cattle, pigs, sheep, goats, and other mammals whose meat may be used as food. The term "poultry" herein includes chickens, turkeys, ducks, quail, and geese. The term "seafood" means any fish or shellfish from the sea used for food; see also Code of Federal Regulation; Title 21, Part 161--Fish and Shellfish. The term "parts thereof" means any part of the carcass which is less than the whole carcass, the part may be cut or removed from the whole carcass; the term "parts thereof" also means any part of seafood which is less than the whole seafood, the part may be cut or removed from the whole seafood.

The term "contacting" herein includes spraying, immersing, dipping, submerging, washing, soaking, and other methods (excluding injecting) of applying (i) a polysulfated polysaccharide, (ii) carboxymethyl cellulose, (iii) guanidine or arginine, optionally with Tween and sodium chloride, (iv) or mixtures thereof to the carcasses or parts thereof. The contacting may be during processing or storage. The contacting time that is effective to fully or partially detach microorganisms (e.g., bacteria) from, or fully or partially inhibit microbial (e.g., bacterial) attachment to, animal or poultry carcasses or seafood or parts thereof is easily determined by one skilled in the art (as shown in the examples below). Generally, polysulfated polysaccharides and

carboxymethyl cellulose require 2-24 hours at 4°C. Generally, guanidine or arginine require a minimum of 30 minutes.

The method of the present invention may be used at any stage in the processing of animal or poultry carcasses or seafood or parts thereof; in addition, the method may be used at different temperatures utilized at different stages in the processing plant. The amount of (i) polysulfated polysaccharides, (ii) carboxymethyl cellulose, (iii) guanidine or arginine, optionally with Tween and sodium chloride, (iv) or mixtures thereof, that is effective to fully or partially detach microorganisms (e.g., bacteria) from, or fully or partially inhibit microbial (e.g., bacterial) attachment to, animal or poultry carcasses or seafood or parts thereof is easily determined by one skilled in the art (as shown in the examples below). Generally, 0.05-2% (preferably 0.1-0.5%) (w/v) polysulfated polysaccharides (e.g., carrageenans), 0.1-0.5% (w/v) carboxymethyl cellulose, 0.1-2M (preferably 0.5-1.0 M) guanidine or arginine, 0.01-25% (preferably 0.05-1%)(w/v) Tween (e.g., Tween 80), and 0.5-3% (preferably 0.85-2) (w/v) sodium chloride may be utilized. These compounds may be dissolved or suspended in 0.05 mM-0.5 M phosphate buffer (sodium or potassium phosphate buffer as a substitute for water).

The animal or poultry carcasses or seafood or parts thereof may be first contacted with a polysulfated polysaccharide or carboxymethyl cellulose and then contacted a second time (1) with a polysulfated polysaccharide or carboxymethyl cellulose or (2) with guanidine or arginine (optionally together with Tween and sodium chloride). The animal or poultry carcasses or seafood or parts thereof may be washed before or after being contacted with the compounds.

Carcass decontamination as spray or immersion bath: The food and animal industry needs a processing method to eliminate or reduce pathogen contamination of foods of animal and poultry and seafood origin. Polysulfated polysaccharides or carboxy-methyl cellulose may inhibit attachment of pathogens from animal and poultry and seafood tissues. In combination with guanidine, arginine and/or Tween and salt, the microorganisms (e.g., bacteria) may be detached from collagen surfaces such as connective tissue, muscle surfaces, fascia, adipose tissues, etc. Polysulfated polysaccharides or carboxymethyl cellulose, in combination with other chemicals, may be used as ingredients in carcass sprays or immersion baths prior to or after

chilling of eviscerated slaughtered food animals and poultry and seafood. When combined with bactericidal agents, for example cetylpyyridium chloride, it may also be used to decontaminate cut parts prior to packaging. Treatment of carcasses and parts thereof with the polysaccharides (carrageenans or carboxy methyl cellulose) may also prevent dehydration of the animal and poultry and seafood parts, thus prolonging their quality and providing economic savings (through prevention of water loss in the chilling process) to the industry.

For example, in beef slaughter, the polysulfated polysaccharides or carboxymethyl cellulose may be dispensed by spraying the carcasses after the post-skinning wash and before evisceration. After splitting of the carcass, microbial (e.g., bacterial) contamination may be removed with the final wash containing arginine, phosphate buffer and Tween/NaCl followed by hot water spray. Polysulfated polysaccharide or carboxymethyl cellulose may be applied the second time to cut up parts prior to packaging and distribution (Figure 1).

For example, in poultry processing, the polysulfated polysaccharides or carboxymethyl cellulose may be dispensed through spraying or immersion in a bath to coat the poultry carcasses after the chilling process. With cut up poultry carcasses, the pieces may be coated by the polysulfated polysaccharide or carboxymethyl cellulose prior to packaging (Figure 2).

For example, in pork processing, the carcass may be sprayed with polysulfated polysaccharides or carboxymethyl cellulose prior to chilling (air blast at 2°C). The coating of the carcass with polysulfated polysaccharides also prevents dehydration of the carcass, thus preserving its quality and providing economic savings to the industry caused by the drying effects of the air blast. The cut pieces of pork carcasses may also be coated by polysulfated polysaccharides or carboxymethyl cellulose prior to packaging. Bactericidal agents (e.g., cetylpyyridium chloride) may also be incorporated with the polysulfated polysaccharides or carboxymethyl cellulose (Figure 3).

In cases where the carcasses have been contaminated, a 2-step processing may be used to detach the microorganisms (e.g., bacteria). First the carcasses may be sprayed or rinsed with polysulfated polysaccharides or carboxymethyl cellulose to destabilize the microorganisms (e.g.,

bacteria) and this may be followed by spraying with arginine or phosphate buffer and Tween/NaCl to remove the destabilized microorganisms (e.g., bacteria).

The present invention also includes a method of quantifying microorganisms (e.g., bacteria) on carcasses or parts thereof. To enhance detachment of microorganisms (e.g., bacteria) for sampling: Treatment of animal and poultry carcass or seafood with polysulfated polysaccharides or carboxymethyl cellulose followed by guanidine or arginine or phosphate buffer and Tween/NaCl may enhance detachment of microorganisms for sampling and analytical purposes. Guanidine-HCl is more effective than arginine for detachment, but the former is not a food additive and may be toxic, though it can be used for analytical (e.g., detection or quantitation) purposes. At pH 4.8, live bacteria may be recovered with guanidine-HCl. At pH 2.5, guanidine-HCl is bactericidal. Therefore, its use may be limited in the laboratory (Figure 4). For "in situ" sampling in the slaughter plants, arginine, or phosphate buffer and Tween 80/NaCl may be used in place of guanidine (Figure 5).

Carrageenan and related compounds (polyanionic polysaccharides) are natural substances and are "generally regarded as safe" (GRAS). These compounds are already used in the food industry for thickening, gelling, stabilizing, water holding property, etc. These compounds are produced worldwide and are inexpensive. Such compounds have not been used for pathogen control in the food industry. The price is $3 to $6 per pound depending on the purity. However, various grades of preparation may have varying effects. The use of carrageenans for pathogen control would be less than 1% as an ingredient for carcass wash water treatment. Carrageenans are compatible with chilled carcasses or hot water sprays. The value of commercial slaughter is $51 billion for animal packing and $28 billion for poultry slaughter and processing, thus the additional cost for use of these compounds would be minimal.

Carrageenans are polysulfated polysaccharides with polymers of repeating D-galactopyranose disaccharides (Glicksman, M., 1983, *Food Hydrocolloids*, Vol 2: 73-113, CRC press, Inc. Boca Raton, Florida.). They are extracted from marine red algae (seaweeds) and used as food additives which are classified as "generally regarded as safe" (GRAS) by the Food and Drug Administration. Heparin sulfate is a polymer of repeating disaccharides of glucoronate

and N-acetylglucosamine. These compounds were used to study inhibition of bacterial attachment to beef fascia, connective tissues and poultry skin and in the design of methods to control contamination of carcass surfaces with pathogens. Figure 6 shows the structures of heparin sulfate and the sulfated polygalactans (carrageenans). Heparin, heparin sulfate and dextran sulfate are also examples of polysulfated polysaccharides; heparin and heparin sulfate would generally not be used for food purposes.

Carrageenans are defined by the Food and Drug Administration according to their sources and usage and applications (CFR Title 21). Carrageenans are refined hydrocolloid extracted from the families of *Gigartinaceae* and *Solieriacea* of the class *Rodophyceae* (red seaweed); *Chondrus crispus*, *Chondrus ocelalatus*, *Euchema cottonii*, *Euchema spinosum*, *Gigartina acicularis*, *Gigartina pistillata*, *Gigartina radula* and *Gigartina stellata*. The food additive contains sulfated polysaccharide with dominant hexose of galactose and anhydrogalactose. Carrageenans contain sulfates in a range of 20-40% dry weight. Salts of carrageenans include ammonium, calcium, potassium or sodium salts. Carrageenans are used as food additives (e.g., emulsifier, stabilizer or thickener in foods). Polysorbate 80 (Tween 80) is used with carrageenan production to facilitate separation of sheeted carrageenan salts of carrageenan from drying rolls. Furcellarans are also polysulfated hydrocolloid from a subtype of *Rodophyceae* (red seaweed) which contains 8-19% sulfates, dry weight. Other sources of carrageenans are *Gloiopeltis*, *Iridaea*, *Furcellaria fastigiata* (Belitz, H.-D., and Grosch, W. (1986), Carbohydrates IN: Food Chemistry, Springer Verlag, New York, NY, pp.201-256), *Hypnea musciformis, Gigartina skottsbergii* (Rees, D.A., (1963), The carrageenan system of polysaccharides, part I the relation between the κ– and λ– components, London J. Chem. Soc., Part II: 1821-1832; Cerezo, (1967), London J. Chem. Soc. (C), 992). The structures, chemistry, functions, applications and food additive uses of carrageenans and polysulfated polygalactans are described by Picullell (Picullell, L. (1995), Gelling Carrageenan, IN: Food Polysaccharides and their applications, (A.M. Stephen, Editor), Marcel Dekker, Inc., pp. 205-244; Belitz, H.-D., and Grosch, W. (1986), Carbohydrates IN Food Chemistry, Springer Verlag, New York, NY, pp.201-256), *Hypnea musciformis, Gigartina skottsbergii* (Rees,

D.A., (1963), The carrageenan system of polysaccharides, part I the relation between the κ– and λ– components, London J. Chem. Soc., Part II: 1821-1832; Glicksman, M. (1983), *Food Hydrocolloids*, Vol 2: 73-113, CRC press, Inc., Boca Raton Florida; Rees, D.A. (1961), Estimation of the relative amounts of isomeric sulphate esters in some sulfated polysaccharides, London J. Chem. Soc., Part IV: 5168-5171; Rees, D.A. (1963), The carrageenan system of polysaccharides, part I the relation between the κ– and λ– components, London J. Chem. Soc., Part II: 1821-1832; Guthrie , R.D., et al. (1968), Carbohydrate Sulphates, IN: Carbohydrate Chemistry, The Chemical Society Burlington House, London, pp. 254-269; Kroschwitz, J.E, et al., Eds. (1994), Encyclopedia of Gum Technology, Volume 12, John Wiley and Sons, New York, NY; Dea , I.C.M. (1982), Polysaccharide conformation in solutions and gels, IN: Food Carbohydrates (D. R. Lineback and G.E. Inglett, Editors), Avi Publishing Co., Westport Connecticut, pp. 420-457).

The polysulfated and polyanionic polysaccharides (such as carrageenans, dextran sulfate, heparin sulfate, carboxymethylsaccharides, alginates) are useful as Microbial Blocking Agents (MBAs) and Surface Rinse Materials (SRMs) for any food product susceptible to microbiological contamination. Representative products include processed or unprocessed animal and poultry products and Ready to Eat (RTE) foods for human consumption or as part of animal feeds, seafood or aquaculture, and in processed and unprocessed seafood products. The polysulfated and polyanionic polysaccharides are particularly useful in treating whole carcasses or parts thereof, ground meat including red meats, pork, and poultry products to block microbial attachments and detach pathogens from these products.

The polysulfated and polyanionic polysaccharides are effective against microorganisms including bacteria, viruses, protozoa and parasites, these materials are specially useful as intervention agents to prevent contamination and proliferation of human food-borne pathogens and food spoilage microorganisms. Examples of microorganisms that can be detached from animal or poultry carcasses or seafood or parts thereof, or whose attachment to animal or poultry carcasses or seafood or parts thereof may be inhibited, generally include the following: Enteric gram-negative pathogens of *Escherichia coli* (enteropathogenic, enterohemolytic, and

enterotoxigenic *E. coli,* especially *E. coli* O17:H7), *Salmonella* species (*S. typhmurium*, *S. enteredities*, *S. dublin*, *S. hartford*, *S. panama*, etc., especially *S. typhimurium*), *Yersinia* species (*Y. enterocolitica*), *Campylobacter* species (*C. jejuni*, *C. colerasuis*), *Shigella* species (*S. dysenteriae*), *Aeromonas* species (*A. hydrophila*), *Vibrio* species (*V. parahemolyticus*, *V. chlorera*); Gram-positive pathogens such as *Staphylococcus* species (*S. aureus*, *S. epidermidis*), *Listeria* species (*L. monocytogenes*); *Helicobacter* species (*H. pylori*), *Bacillus* species (*B. subtilis*, *B. cereus*); viruses such as Hepatitis (A), Norwalk and Norwalk-like viruses, Human immunodeficiency virus (HIV), Herpes virus (H. simplex); parasites such as *Chlamydia* species, *Sarcina* species; Protozoa and fungi and yeasts such as Giardia, Cryptosporidum, Entamoeba (*E. histolyca*), *Candida* (*C. albicans*), Trichomonas (T. vaginalis); spoilage microorganisms such as *Pseudomonas* spp., *Bronchothrix thermosphacta*, *Acinetobacter* spp., *Aerobacter* spp., *Enterobacter* spp., *Moraxella* spp., *Lactobacillus* spp, *Flavobacterium* spp.

The polysulfated and polyanionic polysaccharides are applied at any time during slaughter operations and in the processing of the meat, poultry and seafood products. For example, in the meat slaughter operation after removal of cow hide and prior to evisceration and/or after sanitizing treatment, after cutting and in treatment of the comminuted meat products during and after grinding, and in processing into various products. The MBAs and SRMs can also be applied in the poultry slaughter operation, during carcass washing, preferentially after sanitizing, after cutting up of the poultry into parts and during grinding of the muscle parts into comminuted raw (uncooked), cooked and Ready to Eat products. The inventive material can also be used to treat molluscs or shellfish prior to cultivation and in a depuration process to purge pathogenic microorganisms from the seafood product.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention as defined by the claims.

Experimental Procedures and Results:

Biosensor Studies: A novel approach to study bacterial attachment to animal carcass was designed. The studies led to a development of a biosensor method to study binding of extracellular matrix components to the surface of immobilized bacteria. This novel approach utilized immobilized whole *E. coli* cells. This approach allowed the study of the binding of the lipopolysaccharides and other bacterial surface epitopes without altering their chemical structure. Conditions for immobilization and determination of the bacterial cell surface binding properties were initially assessed with anti-*E. coli* 0157:H7 antibody (Medina et al. (1997), Real-time analysis of antibody binding interactions with immobilized E. coli O157:H7, Biotechnology Techniques, Vol. 11 (3): 173-176). The bacterial surface was bound with an antibody produced against a heat-killed bacteria. A model system was developed to study attachment or binding of biochemically unaltered extracellular matrix and carcass macromolecules such as collagen I, laminin, fibronectin and glycoaminoglycosides (Medina, M.B., and P.F. Fratamico (1998), Binding interactions of collagen I, laminin and fibronectin with immobilized E. coli 0157:H7 using surface plasmon resonance biosensor, Biotechnology Techniques, Vol. 12 (3): 235). Using the binding of collagen-laminin to *E. coli* surface, this system was then utilized for binding studies of the carcass macromolecules and inhibition of their binding to *E. coli* surfaces by carrageenans, polysulfates and other polyanionic gums (Medina, M.B. (1998), Mechanisms of Enterogenic Bacterial Attachment and Inhibition of *E. coli* O157:H7 Binding to Extracellular Matrix Proteins and Tissues, Presented at the 1998 Joint PAASE, DOST and UP Conference, Manila Philippines (Abstract); Medina, M.B. (1998), Biosensor Studies of Collagen and Laminin Binding with Immobilized *Escherichia coli* O157:H7 and Inhibition with Naturally Occurring Food Additives, Presented in SPIE's International Symposium on Industrial and Environmental Monitors and Biosensors: Pathogen Detection and Remediation for Safe Eating (EB20), Nov. 1-6, 1998, Boston, Massachusetts (Abstract); Medina, M. B. (1998), Biosensor Studies of Collagen and Laminin Binding with Immobilized Escherichia coli O157:H7 and Inhibition with Naturally Occurring Food Additives, Proceedings of SPIE-The International Society for Optical Engineering, pp.97-104). The binding interactions of polysulfated polysaccharides such as

heparan sulfate and iota, lambda and kappa carrageenans were determined with the extracellular matrix proteins (ECM) and the immobilized *E. coli* O157:H7 cells. The carrageenans inhibited and mediated the detachment of the macromolecules from the bacterial surface. Without being bound by theory, these properties are presumably due to the biochemical interactions with collagen and laminin, thus blocking the binding of adhesins from the bacterial surface. This biosensor technique was used to assess the binding properties of collagen, laminin, fibronectin, actin and myosin with the surfaces of immobilized *Salmonella typhimurium* ATCC 14028 and DT104 H3380.

Carrageenans and heparan sulfates are polysulfated polysaccharides (Figure 1). Carrageenans are polymers of repeating D-galactopyranose disaccharides (Glicksman, M. (1983), *Food Hydrocolloids*, Vol 2: 73-113, CRC press, Inc., Boca Raton, Florida.). They are extracted from marine red algae (seaweeds) and used as food additives which are classified as "generally regarded as safe" (GRAS) by the Food and Drug Administration. Heparan sulfate is a polymer of repeating disaccharides of glucoronate and N-acetylglucosamine. The carrageenans inhibited and detached the macromolecules from the bacterial surface. Without being bound by theory, these properties are presumably due to biochemical interactions with collagen and laminin, thus blocking the binding of adhesins from the bacterial surface. Optical and electron microscopy studies also show the pattern of inhibition. It has been demonstrated that the *E. coli* O157:H7 bound and attached to collagen as shown in biosensor and electron microscopy studies (Medina 1998).

Collagen is the major component (40%) of the connective tissues. Results from the scanning electron microscopy studies illustrated the abundance of collagen strands in the fascia and connective tissues (Medina et al. (1999), Scanning Electron Microscopy Studies on Attachment of *Escherichia Coli* O157:H7 to Bovine Tissues, Presented at 1999 IFT Annual Meeting, Chicago, IL, July 24-28; Poster at Gordon Research Conference on Molecular Mechanisms of Microbial Adhesions (August 1-6, 1999) and at the 18th Annual Meeting of the Philippine-American Academy of Science and Engineering, Jekyll Is., GA, Aug. 13-15, 1999). This study also showed the attachment of bacterial cells to single strands of collagen fibril. The

fat globules were also wrapped with collagen fibrils where the bacteria attached. The number of bacterial cells were more abundant in crevices of the inoculated tissues. Carrageenans and other structurally related food additives prevented binding of *E. coli* O157:H7 to collagen and reduced attachment of *E. coli* O157:H7 to bovine connective tissues. The binding and dissociation properties of these carrageenans were shown with collagen. Without being bound by theory, the proposed mechanism for its inhibition is perhaps due to competitive binding of these food additives with LPS and other bacterial surface epitopes. If a carcass surface is treated first with these compounds, bacterial LPS and other surface epitopes can not bind to the carcass. The initial phases of bacterial attachment result from the interactions of collagen, LPS and O antigen. However, these food additives showed weak bactericidal effect shown by plating in TSP or BHI media or Buttterfield's buffer. The inhibition results from these biosensor studies are shown in Tables 1, 2a, 2b, and 3; carrageenans were from Ingredient Solutions (formerly Shembreg USA), Searsport, Maine.

Table 1: Biosensor studies showing % Inhibition of collagen-laminin binding to the *E. coli* surface by polysulfated polysaccharides (100 μg/ml)

Inhibitors	Trial 1 % Inhibition	Trial 2 % Inhibition	Trial 3 % Inhibition	Trial 4 % Inhibition	Mean % Inhibition	Sample Standard Deviation
Heparin-SO_4	40	41	35	41	39	2.87
iota-Carrageenan	64	81	91	79	79	11.15
λ-Carrageenan	99	97	99	97	98	1.15
κ-Carrageenan 104	73	67	73	69	71	3
κ-Carrageenan 888	96	88	96	88	92	4.6

Table 2a. Typical binding interactions of food additives with collagen and *Salmonella typhimurium* Sensor Surface.

Sample Injected	Bound RU	% Bound to *ST* sensor	% Inhibition
Collagen	1850	100	-
+PBS	0.2	0	-
+Kappa-888	11	0.5	**99.5**
+Kappa-104	133	6	**94**
+Kappa-C8	180	8	**92**
+Kappa-C1	129	6	**94**
+Alginate,Na	1074	50	**50**
+Pectin, LM	1766	82	**18**
+Dextran SO_4	12.1	0.6	**99.4**

RU= response units or signals from collagen bound to the bacterial sensor. Polysaccharide: Collagen ratio=1:2 (50 μg/ml polysaccharides in 100 μg/ml collagen). % Bound is binding RU of collagen/polysaccharide mixtures / binding RU of collagen. % inhibition is 100 - % bound x 100.

Table 2b. % Inhibition of collagen I binding to immobilized *Salmonellla typhimurium* by kappa-carrageenans and other polysaccharides.

Polyscharides	Collagen: Polysaccharides (50 : 100 μg/ml)	Collagen: Polysaccharides (100 : 50 μg/ml)	Collagen: Polysaccharides (50 :10 μg/ml)
Kappa-8	100	99.5	67
Kappa-1	100	95.9	61
Kappa-C8	99.6	91.7	52
Kappa-C1	100	94	63
Alginate, Na	79	50	17
Pectin, LM	0	18	8
Dextran SO_4	98	99.4	97

Kappa-1, -8, -C8, -C1 are various preparations of food grade kappa-carrageenan. The ratio of collagen and polysaccharides tested were 1:2, 2:1 and 5:1. % inhibition = 100 - % bound x 100 where % Bound is binding RU of collagen/polysaccharide mixtures / binding RU of collagen

Table 3: Biosensor studies showing % Inhibition of collagen binding to bacterial surfaces by various gums and hydrocolloids

	Symbols (not standard)	*E. coli* O157:H7	*Salmonella typhimurium*	*Salmonella typhimurium* DT104
Dextran Sulfate	DS	100	99.4	not tested
Alginic Acid, Na form	SA	50	50	not tested
Low Methoxy Pectin	PLM	5	18	not tested
Agar (non-sulfated polygalactan)	NSA	54	74	62
Gum arabic	GA	5	30	42
Keltone (sodium alginate)	KSA	36	77	70
Tragacanth 1	TG1	0	0	2
Tragacanth 2	TG2	16	12	8
Locust bean gum	LBG	13	--	11
Xanthan	XG	29	17	20
Gum karaya	GK	18	18	11
Polygalacturonic acid	PG	35	--	37
Carboxymethyl cellulose	CMC	84	89	85
Heparan sulfate (bovine intestinal mucosa)	INH	40		
Heparan sulfate (bovine kidney)	INH	38		

In Table 3 are more data on the ability of other polysaccharides and other compounds to inhibit or detach the pathogens from collagen. The results obtained by the BIAcore studies have unexpectedly been shown to be also effective in tissues. The BIAcore studies provide a model

system to rapidly assess the effectiveness of food additives and other compounds for inhibition and detachment of bound pathogens. The results are reported as relative % inhibition of collagen binding to bacterial surface compared to equal concentration of kappa carrageenan are indicated below. See also Palumbo, S.A.,and M. B. Medina (1998), "Quantitative determination of pathogen reduction during animal slaughter and food processing to provide the scientific basis of HACCP and risk assessment", 1998 Progress Report on Food Safety Research Conducted by ARS, ARS-USDA, Beltsville, MD, December 1998, pp. 90-100; Luchansky, J. B., S. Palumbo, and M. Medina (1999), "Quantitative determination of pathogen reduction during slaughter and food processing to provide the scientific basis of HACCP and Risk Assessment", 1999 Progress Report on Food Safety Research Conducted by ARS, ARS-USDA, Beltsville, MD, December 1999, pp. 91-94.

The inhibitory responses of various preparations and sources of kappa-carrageenan showed 89-99.9 % inhibition of collagen binding to the *E. coli* O157:H7 surface. In contrast, the sodium alginate (SA) and low methoxy pectin (PLM) inhibited only 43 and 5% respectively. In this study, the affinity constants of collagen to *E. coli* and *Salmonella* binding in 12 trials (each) ranged from 10^7 to 10^8 (M^{-1}) with a mean of 3.0×10^8 (M^{-1}) for *E. coli* and 2.75×10^7 (M^{-1}) for *Salmonella*. These compounds (polysaccharides) had no direct binding to the bacterial surface but bound to an immobilized calf skin collagen. The dissociation constants of bacteria and collagen binding ranged from 10^{-8} to 10^{-9} (K_DMole) while kappa-carrageenans and collagen binding had typically 10^{-10} (K_DMole). These kinetics indicate that the carrageenans compete for the same binding sites on the collagen surface and their binding is also much tighter or stronger than the bacteria-collagen binding. These polysaccharides could compete with the polysaccharides, such as LPS, on the bacterial surface epitopes.

Tissue *In Vitro* Studies:

Detachment Protocol:

Cut Samples (beef fascia or chicken skin, 4 x 4 cm)

Decontaminate with 5% H_2O_2

Inoculate with *E. coli* to beef fascia or *Salmonella* to chicken skin 3 h at 4°C

Wash off excess unbound bacteria with 3 x sterile H_2O

Plate and count 3rd wash

Detach bacteria with 0.2% INL or 0.3% INK

Plate and count and treat with 0.75M guanidine HCl pH 4.8 (2 x) for final detachment

<u>Inhibition Protocol:</u>

Cut Samples (veal fascia or chicken skin, 4 x 4 cm)

Decontaminate with 5% H_2O_2

Coat tissues with inhibitor (0.2% L-carrageenan or 0.3% K-carrageenan) overnight at 4°C

Squeeze out excess inhibitor

Inoculate with *E. coli* to beef fascia or *Salmonella* to chicken skin for 3 h at 4°C

Wash off excess unbound bacteria 3 x with sterile H_2O

Plate/count 3rd wash

Detach bacteria with 0.2% L-carrageenan or 0.3% K-carrageenan)

Plate and count and further wash with 0.75M guanidine HCl pH 4.8 (2 x)

Plate and count

Veal Fascia Tissue Analysis:

Results on Inhibition of *E. coli* O157:H7 attachment by carrageenan and detachment with guanidine HCl

Sample test	Detachment	Inoculum/ extract	Wash 2 CFU/ml	INL Detached	Guan.
1. PBS (Blank)	PBS-Guan.	0	0	0	0,0
2. *E. coli*	PBS-Guan.	5.8×10^2	0	4.2×10^2	0, 0
3. *E. coli*	INL-Guan.	6.2×10^2	18.5	73	0, 0
4. *E. coli*	INL-Guan.	1×10^3	0	0	0, 0
5. INL; *E. coli*	INL-Guan.	7.5×10^2	0	0	0, 0

PBS= Phosphate buffer; PBS-Guan = Phosphate buffer followed by guanidine wash; INL= λ-carrageenan; INL-Guan.= λ-carrageenan followed by guanidine wash;

Samples were inoculated with 10^{-5} *E. coli* 0157:H7 (9×10^3 cfu/ml).
Two log CFU were washed off.
One log CFU remained in tissue (see inoculum/extract).
Some bacteria were washed off in the first wash.
Sample 3 had remaining bacteria in Wash 2.
Attached *E. coli* was removed (detached) in samples 2 and 3 after INL treatment.
No *E. coli* was recovered in sample #5 pretreated with INL and washed with PBS and guanidine.
No *E. coli* was recovered in guanidine HCl wash.

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Chicken Skin Tissue Analysis

Results on Inhibition/Detachment of *Salmonella typhimurium* ATCC 14028 (ST) inoculated on chicken skins.

Sample Test	Detachment Cpd	Detached CFU	Guanidine, pH 2.5
1. ST	PBS	26, 52	0, 3
2. ST	PBS	16, 27	0, 1
3. ST	INL-Guan.	21, 8	10, 10
4. ST	INL-Guan.	12, 10	0, 25
5. INL; ST	INL-Guan.	27, 20	4, 0
6. INK; ST	INL-Guan.	9, 8	4, 0

PBS= Phosphate buffer; PBS-Guan = Phosphate buffer followed by guanidine wash; INL= λ-carrageenan; INL-Guan.= λ-carrageenan followed by guanidine wash; INK = κ-carrageenan 888.

Samples were inoculated with 10^5 *Salmonella typhimurium* (18 hr culture).
Each reported CFU is an average of two plate counts and represents actual counts.
More CFUs were detached by INL-guanidine detachment than PBS alone.
(Second guanidine detachment had 0 bacteria.)
Samples #5 and #6 were pretreated with INL and INK respectively. INK inhibited attachment of ST to poultry skin. INK (0.3%) is more effective than INL (0.2%).
Inhibitors directly bind to collagen and prevent bacterial binding. Same CFUs in PBS and INL treatment. Actual detachment is facilitated by guanidine -HCl.

Use of Guanidine, creatine and arginine: Guanidine is a strong cytotoxic compound and can not be used in large quantities in food plants or slaughter operations. It is a very effective detachment agent to detach the bacteria bound to collagen. However, its use may be limited in the laboratory for analysis of food tissues. Therefore, a substitute for guanidine for detachment of pathogens from food carcasses was investigated. Guanidine is structurally related to creatine and arginine with a guanidyl moeity in their molecules. However, with the biosensor studies, creatine proved ineffective. It has been shown that creatine hydrolyzes upon dissolution in water

or buffer converting it to creatinine (methylglycocyamidine), a cyclic product and an end product of creatine metabolism. In contrast, 0.5M arginine was equally good or better than 1 M guanidine-HCl to detach the bacteria from the collagen complex. The biosensor studies showed that its effectiveness to detach bacteria bound to collagen is enhanced with the use of Tween 80 and NaCl. Tween 20 and NaCl only partially detached the bound bacteria from collagen. Arginine is an essential amino acid for children but not for adults. It is non-essential for adults because it can be derived from proteins in the diet. It is also inexpensive and can be obtained in bulk. Phosphoric acid (0.1M) , HCl (0.1M) and citric acid (0.5M) had no effect in the detachment studies.

Data on Detachment of *E. coli* O157:H7 from Inoculated Bovine Tissues

Experimental Procedure:

1. Beef bottom round was thinly sliced (approx. 2 mm thick) and cut up into 5 x 5 cm.
2. Veal fascia and connective tissues were separated from veal breast and cut up into 5 x 5 cm.
3. *E. coli* O157:H7 was grown in 25 ml BHI broth at 37°C, overnight (16 hrs).
4. The bacteria was diluted up to 10^{-3} (approx. 10^{6} CFU) for inoculation.
5. The bovine tissues were decontaminated with 20 ml 5% hydrogen peroxide for 30 min.
6. The excess peroxide was squeezed off and the tissues were transferred to stomacher bags containing to 50ml sterile water.
7. The excess water was squeezed off from the tissues and transferred to stomacher bags containing 20 ml of peptone buffer.
8. *E. coli* inoculum (0.1 ml) diluted to 10^{-3} was transferred to tissue samples.
9. The *E. coli* were allowed to attach to the tissues for 30 min at room temperature.
10. The bacterial population in tissue inoculum was plated and counted.
11. The tissues were transferred to stomacher bags containing 50 ml sterile water to rinse off excess bacteria.
12. The bacteria was detached using the detachment procedure outlined in the following tables.

13. The detachment procedures used 25 ml of the combination of water, polysaccharides (INK=k-carrageenan, CMC=carboxymethylcellulose, DS=dextran sulfate), 0.05% Tween 80 (same as SPAN 80) mixed with 0.125N (0.9%) sodium chloride and 0.75M (15%) arginine (food grade substitute of guanidine)

<u>Number of samples analyzed:</u> Each treatment had duplicate tissue samples. The culture for inoculation and the unattached bacteria in inoculated samples were plated in three dilutions on duplicate BHI agar.

<u>Detachment Procedures:</u> The bacteria was detached by the combination of water, polysaccharides, arginine, Tween/ NaCl were plated in 0 to 2 dilutions on duplicate BHI agar.

Sample: Beef Bottom Round

Trail I Inoculum: 1.42×10^6 CFU, No. of analysis (n)= 6; Trial II Inoculum: 2.47×10^6 CFU, n=6; Trial III Inoculum: 2.07×10^6, n=6

	Trial I	Trial II	Trial III
Detachment	CFU[1]	CFU[2]	CFU[3]
Procedure I			
Water-Tween 80/NaCl	2.73×10^2	3.12×10^2	4.11×10^2
INK-Tween 80/NaCl	2.80×10^2	3.38×10^2	1.76×10^2
CMC-Tween 80/NaCl	6.41×10^2	5.36×10^2	6.94×10^2
DS- Tween 80/NaCl	1.04×10^3	3.11×10^2	1.47×10^2
Tween 80/NaCl (2x)		2.66×10^2	6.70×10^2

[1]Highest detachment with DS followed by Tween/NaCl (DS-Tween80/NaCl), then by CMC followed by Tween/NaCl>INK>water + Tween/NaCl
[2] Highest detachment with CMC followed by Tween/NaCl (CMC + Tween/NaCl).
[3]Highest EC detachment with CMC + Tween/ NaCl and 2x Tween/NaCl. Lower EC recovery in INK and DS + Tween/NaCl.
CMC and Tween/NaCl show the highest detachment but the difference is less than one log.

Sample: Beef Bottom Round Inoculum: 5.28×10^5 CFU, n=6

	Trial I
Detachment Procedure II	CFU
Water-Tween 80/NaCl	5.18×10^2
INK-Tween 80/NaCl	4.11×10^2
CMC-Tween 80/NaCl	7.85×10^2
DS-Tween 80/NaCl	3.73×10^2
Tween 80/NaCl (3x)	1.10×10^3

Detachment is highest with 3 x Tween/NaCl, then by CMC + 2 x Tween/NaCl >H_2O>INK>DS + Tween/NaCl

Sample: Beef Fascia Connective Tissues

Trial I Inoculum: 7.73×10^6 CFU, n=6; Trial II Inoculum: 1.23×10^6 CFU, n=6

	Trial I	Trial II
Detachment Procedure III	CFU[1]	CFU[2]
Water-Tween 80/NaCl (2x)	1.14×10^3	1.33×10^3
INK-Tween 80/NaCl (2x)	1.23×10^3	1.21×10^3
CMC-Tween 80/NaCl (2x)	1.14×10^3	1.41×10^3
DS-Tween 80/NaCl (2x)	1.03×10^3	4.62×10^2
Tween 80/NaCl (3x)	1.43×10^3	8.95×10^3

[1]Three rinses with Tween/NaCl detached the highest number of CFUs, then INK + 2x Tween/NaCl; CMC and H20 + 2x Tween washes; and DS+ 2X /NaCl washes.
[2]Three rinses with Tween/NaCl detached the highest number of CFUs, then CMC + 2x Tween/NaCl > H20 + 2x Tween washes; and INK+ 2XTween/NaCl washes. Again, the differences among the detachment procedures were less than one log CFU.
This detachment procedure (III) seem to have the highest detached bacteria which were in the 10^3 levels.

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Sample: Beef Fascia Connective Tissue

Inoculum: 6.54×10^5 CFU, n=6

Detachment Procedure IV	CFU	Detachment Procedure V	CFU[2]
Water-Tween80/NaCl-Arginine	3.32×10^2	Water-Tween80/NaCl-Arginine-Tween/NaCl	3.32×10^2
INK-Tween80/NaCl-Arginine	8.75×10^2	INK-Tween80/NaCl-Arginine-Tween/NaCl	9.13×10^2
CMC-Tween80/NaCl-Arginine	4.30×10^2	CMC-Tween80/NaCl-Arginine-Tween/NaCl	4.61×10^2
DS-Tween80/NaCl-Arginine	3.94×10^2	DS-Tween80/NaCl-Arginine-Tween/NaCl	4.25×10^2
Tween80/NaCl(2x)-Arginine	1.37×10^3	Tween80/NaCl(2x)-Arginine-Tween80/NaCl	1.36×10^3

2 x Tween80/NaCl + arginine + Tween80 had highest detachment, then INK + Tween80 +arginine + Tween80

Sample: Beef Fascia Connective Tissue

Trial I--Inoculum: 3.62 x 10^6 CFU, n=6; Trial II--Inoculum: 3.62 x 10^6 CFU, n=6
Total CFUs in polysaccharide, Arginine, and Tween/NaCl rinses.

	Trial I	Trial II
Detachment Procedure VI	CFU[1]	CFU[2]
Water- Arginine-Tween80/NaCl	1.95 x 10^2	1.64 x 10^3
INK-Arginine-Tween80/NaCl	4.95 x 10^2	5.60 x 10^2
CMC-Arginine-Tween80/NaCl	2.25 x 10^2	1.62 x 10^2
DS-Arginine-Tween80/NaCl	2.30 x 10^2	5.60 x 10^2
Tween/NaCl-Arginine-Tween/NaCl	not inoculated	1.09 x 10^3

[1]INK followed by arginine followed by Tween/NaCl (INK + arginine + Tween/NaCl)detached the most number of CFUs, 2 x higher than with CMC and DS and 2.5 x higher than H_2O. The Tween/NaCl sample was not inoculated.
[2]Tween/NaCl + arginine + Tween detached more CFUs than with additional treatment of INK, CMC and DS.
Again, the differences among the protocol were less than one log CFU.

Sample: Beef Fascia Connective Tissue:

Trial III--Inoculum: 1.905 x 10^6 CFU, n=6; Trial IV--Inoculum: 1.23 x 10^6 CFU, n=6
Total CFUs in polysaccharide, Arginine, and Tween/NaCl rinses.

	Trial III	Trial IV
Detachment Procedure VII	CFU	CFU
Water- (3X)	2.01 x 10^2	9.71 x 10^2
INK-Arginine-Tween/NaCl	1.53 x 10^2*	8.9 x 10^2
Tween/NaCl (3X)	3.19 x 10^2	2.32 x 10^3

*In Trial III, one duplicate sample of the INK treated tissue had no CFUs in the water wash and arginine rinses. In Trials III and IV, rinses with Tween/NaCl yielded the highest number of CFUs/ml detached.

Summary of Results:

Tissues inoculated with 10^6 CFU of E. coli O157:H7, retained 10^2 to 10^3 CFU.

E. coli (10^4) remained in the inoculum and were rinsed off in the water wash.

The detachment procedures used the combination of water, polysaccharides (INK=k-carrageenan, CMC=carboxymethylcellulose, DS=dextran sulfate), 0.05% Tween 80 (same as SPAN 80) mixed with 0.125N (0.7%) sodium chloride and arginine (food grade substitute of guanidine) detached 10^2 to 10^3 CFU of the bacteria.

CMC-Tween/NaCl; 3x Tween/NaCl/INK-arginine-Tween/NaCl; Tween/NaCl-arginine-Tween/NaCl showed higher detachment of the bacteria. However, the differences were less than one log CFU.

With the use of arginine, detachment effect of INK was enhanced (see Detachment Procedures IV-VI)

Any of the combinations described in the summary table may be used for detachment and may be further optimized to effect maximum detachment.

Longer exposure of contaminated tissues to the detaching agents may improve detachment efficiency.

In Trials 1 and II, INK followed by arginine followed by Tween/NaCl (INK + arginine + Tween/NaCl) detached the most CFUs, 2 x higher than with CMC and DS and 2.5 x higher than H_2O. The Tween/NaCl sample was not inoculated. Tween/NaCl + arginine + Tween detached more CFUs than with additional treatment with INK, CMC and DS.

Again, the differences in results among the protocols were less than one log CFU.

In Trial III, one duplicate sample of the INK treated tissue had no CFUs in the water, INK and arginine rinses. In Trials III and IV, rinses with Tween/NaCl yielded the highest number of CFUs/ml.

Inhibition and Detachment of *E. coli* O157:H7 from Inoculated Bovine Tissues

Experimental Procedure:

1. Veal fascia and connective tissues were separated from veal breast and cut up into 5 x 5 cm.
2. The bovine tissues were decontaminated with 20 ml 5% hydrogen peroxide for 30 min.
3. The excess peroxide was squeezed off and the tissues were transferred to stomacher bags containing to 50 ml sterile water.
4. The excess water was squeezed off from the tissues and transferred to stomacher bags containing 20 ml inhibitor solutions. [1-water; 2-0.3% INK; 3-0.3% CMC, 4-0.3% DS; Tween/NaCl. (INK=k-carrageenan, CMC=carboxymethylcellulose, DS=dextran sulfate)
5. *E. coli* O157:H7 was grown in 25 ml BHI broth at 37°C, overnight (16 hrs).
6. The bacteria was diluted up to 10^{-3} (approx. 10^{5-6} CFU) in buffered peptone for inoculation.
7. The excess inhibitors were squeezed off from the tissues and the latter were transferred to stomacher bags.
8. 20 ml of *E. coli* inoculum (prepared by diluting 0.1 ml of original broth to 10^{-3})was transferred to tissue samples.
9. The *E. coli* was allowed to attach to the tissues for 20 min at room temperature followed by overnight attachment at 4°C.
10. The bacterial population in tissue inoculum was plated and counted.
11. The tissues were transferred to stomacher bags containing 50 ml sterile water to rinse off excess bacteria.
12. The bacteria was detached by immersing the tissues sequentially in 50 ml 15% arginine, and 0.05% Tween 80 (same as SPAN 80) mixed with 0.9% sodium chloride.
13. The bacterial counts were measured in arginine and Tween/NaCl by plating in BHI agar.
14. To determine if the tissues contain residual bacteria and *E. coli* O157:H7, the tissues were cultured in 25 ml of modified *E. coli* (mEC) broth (FSIS defined), incubated overnight at 37°C
15. The mEC cultures were plated and counted on either BHI or SMAC agars.

<u>Number of samples analyzed:</u> Each treatment (trials) had duplicate tissue samples. The culture for inoculation and the unattached bacteria in inoculated samples were plated in three dilutions on duplicate BHI agar.

INHIBITION--Trial 1 Sample: Veal connective tissue Inoculum = 4.3×10^6 (n=6)

Sample #	Sample Treatment	Excess bacteria in inoculum	Water Rinse	Arginine Detachment	Tween80/NaCl Detachment
1	H_2O-EC-H_2O-Arginine-Tween80/NaCl	2.5×10^3	1	4.63×10^1	0
2*	INK-EC-H_2O-Arginine-Tween80/NaCl	4.93×10^3	2	3.71×10^1	0
3	CMC-EC-H_2O-Arginine-Tween80/NaCl	5.08×10^3	2	4.17×10^1	0
4	DS-EC-H_2O-Arginine-Tween80/NaCl	2.92×10^3	1	3.71×10^1	2.78×10^1
5	Tween80/NaCl-EC-H_2O-Arginine-Tween80/NaCl	21.2×10^3	2	6.95×10^1	1.85×10^1

* One INK duplicate sample exhibited pinpoint growth in cultured mEC broth indicating that microorganisms in tissues were not *E. coli* O157:H7. One CFU of *E. coli* O157:H7 was detected in sample 4b tissue when cultured in mEC (modified *E. coli*) broth (selective for *E. coli*). In all samples (Numbers 1-5) *E. coli* O157:H7 was recovered in water and arginine washes but were not recovered with Tween/NaCl wash in Samples 1, 2 and 3.
EC = *E. coli*
Sample 1 = H_2O-EC-H_2O-Arg-Tween80/NaCl = H_2O followed by EC followed by H_2O followed by Arginine followed by Tween80/NaCl

INHIBITION--Trial 2 Sample: Veal connective tissue Inoculum = 8.0×10^6 (n=6)

Sample #	Sample Treatment	Excess bacteria in inoculum	Water Rinse	Arginine Detachment Rinse	Tween80/ NaCl Detachment Rinse	Total Rinses H_2O-Arginine-Tween80/NaCl
1	H_2O-EC-H_2O-Arginine-Tween80/NaCl	1.42×10^2	4.63×10^2	1.40×10^2	5.1×10^1	6.54×10^2
2	INK-EC-H_2OArginine-Tween80/NaCl	1.69×10^4	1.85×10^2	4.17×10^1	3.7×10^1	2.64×10^2
3	CMC-EC-H_2O-Arginine-Tween80/NaCl	1.63×10^4	0	3.71×10^1	2.78×10^1	6.49×10^1
4	DS-EC-H_2O-Arginine-Tween80/NaCl	9.99×10^3	3.69×10^2	1.27×10^2	5.56×10^1	5.52×10^2
5	Tween80/NaCl-EC-H_2O-Arginine-Tween80/NaCl	21.2×10^4	1.85×10^2	7.41×10^1	3.70×10^1	2.96×10^2

Tissues cultured in mEC broth have contaminants in samples 1 - 4. Sample 5a had no bacteria while sample 5b had 1 CFU/ml. *E. coli* was not detected in all samples except in 5b.

INHIBITION--Trial 3, Sample: Veal connective tissue   Inoculum = 4.315×10^6 (n=6)

Sample #	Sample Treatment	Excess bacteria in inoculum	Water Rinse	Arginine Detachment Rinse	Tween/NaCl Detachment Rinse	Total Rinses H_2O-Arginine-Tween80/NaCl
1	H_2O-EC-H_2O-Arginine-Tween80/NaCl	1.18×10^4	0	4.61×10^1	3.71×10^1	8.33×10^1
2	INK-EC-H_2O-Arginine-Tween80/NaCl	7.65×10^3	0	2.31×10^1	0	2.33×10^1
3	CMC-EC-H_2O-Arginine-Tween80/NaCl	3.52×10^3	0	2.77×10^1	1.85×10^1	4.62×10^1
4	DS-EC-H_2O-Arginine-Tween80/NaCl	8.59×10^2	1.85×10^2	6.50×10^1	4.16×10^1	2.92×10^2
5	Tween80/NaCl-EC-H_2O-Arginine-Tween80/NaCl	4.61×10^3	0	7.42×10^1	2.77×10^1	1.02×10^2

Samples treated with INK (Sample 2) and CMC (Sample 3) showed greater inhibition vs those samples treated with water (Sample 1), DS (Sample 4) and Tween/NaCl (Sample 5) shown by the lower number of CFUs recovered in the "Total rinses".

Tissue samples cultured in mEC broth demonstrated no *E. coli* growth in samples 1, 2, 4 and 5. One replicate sample (3a) of sample 3 treated with CMC had one CFU/ml of *E. coli.*

Summary of results in the inhibition/detachment experiments:

These data demonstrate the effectiveness of carrageenans, arginine, Tween80/NaCl treatments.

In Trial 1, one K-carrageenan (INK) duplicate sample exhibited pinpoint growth in cultured mEC broth indicating that microorganisms in tissues were not *E. coli* O157:H7. One CFU of *E. coli* O157:H7 was detected in sample 4b tissue when cultured in mEC broth.

In all samples (Numbers 1-5) *E. coli* O157:H7 was recovered in water and arginine washes but were not recovered with Tween/NaCl wash in Samples 1, 2 and 3.

In Trial 2, tissues cultured in mEC broth have contaminants in samples 1 - 4. Sample 5a had no bacteria while sample 5b had 1 CFU/ml.

In Trial 3, samples treated with INK (Sample 2) and CMC (Sample 3) showed greater inhibition vs those samples treated with water (Sample 1), DS (Sample 4) and Tween/NaCl (Sample 5) shown by the lower number of CFUs recovered in the "Total rinses".

Tissue samples cultured in mEC broth demonstrated no *E. coli* growth in samples 1, 2, 4 and 5. One replicate sample (3a) treated with CMC had one CFU/ml of *E. coli.*

These inhibition and detachment procedures prevented the attachment of most *E. coli* to the veal connective tissues and were detached with sequential rinses in water, arginine and Tween/NaCl solutions.

Inhibition of *Salmonella typhimurium* attachment to chicken skin by the polysaccharides and detachment with water, arginine and Tween 80/NaCl.

Method:

1. Fresh chicken skin was cut into 24-25 cm^2 surface.
2. The tissues were immersed in 5% hydrogen peroxide to inactivate the natural flora and followed by washing in 50 ml water.
3. The samples were transferred to stomacher bags and coated with the following:
   A. Water wash
   B. 0.3% K-carrageenan (INK)
   C. 0.05% Tween 80-0.9% NaCl
   D. 0.3% Carboxymethyl cellulose (CMC)
   E. 0.3% Dextran Sulfate (DS)
4. The samples were transferred to fresh stomacher bags containing 50 ml water to remove excess inhibitors. (this step was used in Trial 3)

5. *Salmonella typhimurium* (ST) was inoculated on the outer skin surface using 0.1 ml of 10^4 cfu/ml and spreading with a "hockey stick" glass rod.

6. ST was allowed to grow and attach for 1 hr at room temperature followed by overnight incubation at 4°C.

7. The samples were transferred to stomacher bags for detachment using the following:

   A. Water wash (3x)

   B. 0.75M Arginine and followed by 2x with 0.05% Tween 80-0.9%NaCl

   C. 0.05% Tween 80-0.9%NaCl (3x)

   D. 0.75M Arginine and 2x 0.05% Tween 80-0.9%NaCl

   E. 0.75M Arginine and 2x 0.05% Tween 80-0.9%NaCl

8. The tissue samples were then plated directly on XLT4 agar with the outer skin side touching the agar surface. Samples were incubated at 37°C overnight.

Results: The tissues were divided into 4 quadrants and the number of quadrants with black spots were reported as + if only one quadrant has black spots, ++ if two quadrants have black surfaces, etc. Treatment of the tissues with INK (kappa-carrageenan) followed by arginine and Tween/NaCl showed negative results in 3 out of four samples compared to water wash, DS (dextran sulfate), CMC (carboxymethylcellulose) or Tween and NaCl washes. These treatments suggest that carrageenan had greater inhibition effects than water wash alone, dextran sulfate/arginine/Tween 80-NaCl or carboxy-methyl-cellulose/arginine/11:20tween-NaCl treatments. See Table 1.

Table 1. Black spots (colonies) on XLT4 agar.

Treatment	Trial 1A	Trial 1B	Trial 2A	Trial 2B
A. Water wash (4x)	++++	+ (trace)	+++	+++
B. INK-Arginine-Tween80	- (neg.)	- (neg.)	+++	- (neg.)
C. Tween80/NaCl (4x)	++++	++++	+	- (neg.)
D. CMC-Arginine-Tween80	+++	+++	++++	+
E. DS-Arginine-Tween80	++	++	- (neg.)	+++

(Black colonies or spots indicate positive for Salmonella. The number of + indicate the number of quadrants if the tissue was segmented to 4 parts.)

Table 1: Trial 3. Black spots (colonies) on XLT4 agar.

Treatment	Trial 3A	Trial 3B
A. Water wash (4x)	+++	+ ++
B. INK-Arginine-Tween80	<+	<+
C. Tween80/NaCl (4x)	++++	+++
D. CMC-Arginine-Tween80	++++	++
E. DS-Arginine-Tween80	++++	+

Black colonies or spots indicate positive for *Salmonella*. The number of + indicate the number of quadrants if the tissue was segmented to 4 parts.)

Treatment of the tissues with INK (kappa-carrageenan) inhibited Salmonella attachment greater than water wash, DS (dextran sulfate), CMC (carboxy methyl cellulose) or Tween80 and NaCl washes. These treatments were followed by arginine and Tween/NaCl).

Figure 7 shows the results from Trial 3A and 3B. The carrageenan treated samples (1a, 1b) followed by arginine and Tween-80 washes showed the least contamination with *Salmonella*. The contamination was only at the edges of the samples. This may be due to the curling of the edges during the test, the edges may not have gotten fully coated with carrageenan. "C" is the control sample and was not inoculated with *Salmonella*.

Detachment of *Salmonella typhimurium* inoculated to pork skin.

1. Fresh pig skin was cut into 24-25 cm^2 surface.
2. The tissues were immersed in 5% hydrogen peroxide to inactivate the natural flora.
3. *Salmonella typhimurium* (ST) was inoculated on the outer skin surface with 0.1 ml of 10^6 cfu/ml.
4. ST was allowed to grow and attach for 1 hr at room temperature followed by overnight incubation at 4°C.
5. The samples were transferred to stomacher bags for detachment using the following:

A. Water wash (4x)

B. 0.3% K-carrageenan (INK), followed by 0.75M arginine followed by 2x with Tween 80-NaCl (INK-ARG-2X-Tween/NaCl)

C. 0.05% Tween 80-0.9%NaCl (4x) (Tween/NaCl 4X)

D. 0.3% carboxymethyl cellulose (CMC); 0.75 arginine; and 2x 0.05% Tween 80-0.9%NaCl (CMC-ARG-2XTween/NaCl)

E. 0.3% Dextran Sulfate (DS); 0.75M arginine; and 2x 0.05% Tween 80-0.9%NaCl (DS-ARG-2XTween/NaCl)

6. The tissue samples were then plated directly on XLT4 agar with the skin side touching the agar surface. Samples were incubated at 37°C overnight.

Results: These results are shown in attached photographs (Figure 8). The results indicated that ST remained on the samples treated with water (4x) and CMC-Arginine-Tween80/NaCl. Samples treated with Tween 80-NaCl alone apparently enhanced the attachment of ST. Samples treated with carrageenan-arginine-Tween/NaCl and Dextran sulfate-arginine-Tween/NaCl apparently detached ST from pork skin surface.

Extraction/Isolation of natural *E. coli*, coliforms and *Salmonella* spp. from ground chicken purchased from a local market.

Sample Preparation

1. Aseptically mix ground chicken by mashing manually to make a homogenous mix.
2. Make a ball and flatten the ground turkey into a round shape on a sterile cutting board.
3. Then cut four ways and transferred into 4 sterile bags. Take the opposite quarters for analysis and store the other quarters at -20°C.
4. Using a sterile spatula weigh out 25g into a stomacher bag.
5. Pre-coat samples (#3 and4) with 25ml 0.3%INK, 30min. at room temperature.
6. Squeeze fluid from chicken and transfer coating fluid to a sterile 250ml flask. Store on ice.
7. Rinse 1 Add the following rinse materials to ground chicken samples and homgenize with a stomacher for 1min at low speed.
    #1ab) 100ml butterfields buffer
    #2ab) 100ml PBST
    #3ab) 100ml PBST
    4ab) 100ml arginine
8. Extract, squeeze fluids and transfer fluids to 250ml flasks. Store on ice.
9. Rinse 2 Add the following rinse materials to ground chicken samples and homgenize with a stomacher for 1min at low speed.
    1ab) 100ml butterfields buffer
    2ab) 100ml PBST
    3ab) 100ml PBST

4ab) 100ml PBST

10. Extract, squeeze fluids and transfer fluids to 250ml flasks. Store on ice.
11. Pool liquids collected in steps 6, 8 and 10.
12. Adjust volume to 250ml with Butterfields Buffer.

Testing for Coliform and *E.coli*

13. Take aliquots of pooled rinses from Samples #1-4 and dilute to 10^{o}, 10^{-1}, 10^{-2}.
14. Test for coliform and *E. coli* using 3M Petrifilm by transfering 1 ml of diluted samples over the gel as directed by the manufacturer.
15. Incubate gel film for 24 hr at 37oC.
16. Examine results for gas producing red (coliform) and blue colonies (confirmed *E. coli*) and blue colonies without gas (presumptive *E.coli*).

Determine detachment of *Salmonella* spp. from ground chicken by culturing in non-selective and selective broth and detection with XLT4 Agar.

Treat samples as in steps 1-12:

Non-selective enrichment for *Salmonella*:

17. Take out 30 ml aliquot of pooled liquid wash separately into four sterile 125 ml flask.
18. Add 30 ml of double strength Buffered Peptone Water (BPW).
19. Incubate flasks at 35oC +/-1°C for 20-24 hr.
20. Dilute inoculum at 10^{-2}, 10^{-3}, 10^{-4} and plate 50 µl of of samples over XLT4 agar.
21. Incubate at 35+/- 1°C for 22-24hrs. Examine for black coloniesor yellow to red wtih black centers [sulfur producing *Salmonella* strains]; yellow-pink indicate the presence of non sulfur producing *Salmonella* strains.
22. Reincubate negative plates. Re-examine next day.

Selective enrichment for *Salmonella* in TT Broth and RV Broth

23. Transfer 0.5ml of samples enriched in non-selective broth to 10 ml of TT broth.

24. Transfer 0.1ml of samples enriched in non-selective broth to 10ml of RV broth.

25. Incubate at 42°C for 22-24hrs.

26. Dilute to 10^{-4}, 10^{-5}, 10^{-6}. Plate on XLT4 Agar in duplicate per sample.

27. Incubate at 35+/- 1°C for 22-24hrs. Examine for black or red colonies.

28. Reincubate negative plates. Re-examine next day.

Detection of *Salmonella Spp.* from ground chicken extracted and isolated by inventive rinse agents

Trial 1	Ground chicken	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-		+
1b	BB 2x	-		+
2a	PBST 2x	-	+	+
2b	PBST 2x	-		+
3a	INK-PBST-PBST	-		+
3b	INK-PBST-PBST			+
4a	INK-Arg-PBST		+	
4b	INK-Arg-PBST	-	+	

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Trial II	Ground chicken	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-		
1b	BB 2x	-	+	+
2a	PBST 2x	-	+	+
2b	PBST 2x	-	+	
3a	INK-PBST-PBST	-	+	
3b	INK-PBST-PBST		+	
4a	INK-Arg-PBST	-		+
4b	INK-Arg-PBST	-		+

Trial III	Ground chicken	*Salmonella spp.*	*Salmonella spp.*	*Salmonella spp.*
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-		
1b	BB 2x	-		
2a	PBST 2x	-	+p	
2b	PBST 2x	-	+P	
3a	INK-PBST-PBST	-		
3b	INK-PBST-PBST		+p	
4a	INK-Arg-PBST	-		
4b	INK-Arg-PBST	-		

Results: These samples did not test positive for *Salmonella* after enrichment of pooled rinse materials in non-selective broth. However, when enriched in selective broths (TT and RV) *Salmonella* spp. were invaiably detected in all test samples in Trials I and II. In Trials 1, these results indicate that *Salmonella* was present in the ground chicken samples as injured cells and were revived in selective enrichment. In Trial III, pink colonies (+p) were detected in samples, 2a, 2b and 3a, indicating presence of non-sulfur producing *Salmonella*. Blank data also indicate no detectable *Salmonella*.

Summary data on detachment of *E. coli* and coliforms from ground chicken and their detection with Coliform/*E. coli* Petrifilm test.

Sample No.	Treatment	Trial I Coliform	Trial I *E. coli*	Trial II Coliform	Trial II *E. coli*	Trial III Coliform	Trial III *E. coli*
1a	BB 2x	64, 9, 1		19, 14, 1	6	41, 4	**13**
1b	BB 2x	56, 2		13, 6, 1	2, 2	35, 5	7, 1
2a	PBST 2x	52, 4		15, 3	2, 1	39, 2	6
2b	PBST 2x	46		80, 1	4, 3	37, 2, 1	1
3a	INK-PBST-PBST	**76,** 1		**160**, 1, 1	5	37, 1	5
3b	INK-PBST-PBST	39, 5		42, 4, 1	2, 2	**48**, 5	9, 2
4a	INK-Arg-PBST	28, 4, 1		13, 3	**31**	4	1
4b	INK-Arg-PBST	36, 2		6	2	1	1

Sample treatment: BB= Butterfield's buffer; PBST= phosphate buffer containing 0.9% sodium chloride and 0.05% Tween 80; INK = kappa-carrageenan; arg= arginine. Each sample was plated

at three dilutions, 10^0, 10^{-1}, and 10^{-2}. One ml sample was applied on Petrifilm. The manufacturer's guidelines and interpretation of results were followed. Results indicated by single numbers are detected at one dilution, typically in undiluted sample washes (10^0). When 2 are reported, the bacteria are detected at 10^0 and 10^{-1}, and 10^0, 10^{-1}. and 10^{-2}, The CFU reported indicate actual number per Petrifilm test (based on 1 ml sample) and are not adjusted to sample dilution. Samples without numbers indicate that coliform or *E. coli* was not detected. The coliforms were indicated by red colonies with gas bubbles. Confirmed *E. coli* were indicated by blue to red colonies with gas bubbles.

These results indicate that the coliform and *E. coli* were randomly distributed in the samples and were detached and isolated by the 4 treatments. However, the highest number of detached Coliforms were detected when the samples were pre-coated with carrageenans and rinsed with PBST. In Trials I and II, the highest number of *E.coli* were detected in Samples 1a and 4a. In Trial III, non-sulfur producing *Salmonella* strains were detected as indicated by pink colonies and pink colonies with cream centers.

Extraction and isolation of natural bacteria and *Salmonella* spp. from ground turkey

Sample Preparation

1. Aseptically mix ground turkey by mashing manually to make a homogenous mix.
2. Make a ball and flatten the ground turkey into a round shape on a sterile cutting board.
3. Then cut four ways and store separately. Take the opposite quarters for analysis.
4. Using a sterile spatula weigh out 25g into a stomacher bag.
5. Pre-coat sample #3 only with 25ml 0.3%INK, 30min. at room temperature.
6. Rinse 1-Add the following rinse materials to ground turkey samples and homogenize with a stomacher for 1min at low speed.

   #1ab) 125 ml Butterfield's Buffer

   #2ab) 125 ml 0.3% INK in BB

   #3ab) 100ml PBST

7. Extract, squeeze fluids and transfer fluids to 250ml flasks. Store on ice.
8. Rinse 2. Add 100 ml of the rinse agents described in Step 6.
9. Extract turkey with a stomacher at low speed for 1min. Transfer and pool liquids as described in step 6.
10. Adjust volume to 225ml with Butterfield's Buffer.

Testing for Coliform and *E.coli*

11. Take aliquots from Samples #1-3 and dilute to 10^{0}, 10^{-1}, 10^{-2}.
12. Test for coliform and *E. coli* using 3M Petrifilm by transfering 1 ml of diluted samples over the gel as directed by the manufacturer.
13. Incubate gel film for 24 hr at 37°C.
14. Examine results for gas producing red (coliform) and blue colonies (confirmed *E. coli*) and blue colonies without gas (presumptive *E.coli*).

Determine detachment of *Salmonella spp.* from ground turkey by culturing in non-selective and selective broth and detection with XLT4 Agar.

Treat samples as in steps 1-10:

Non-selective enrichment for *Salmonella*:

15. Take out 30 ml aliquot of pooled liquid wash separately into four sterile 125 ml flask.
16. Add 30 ml of double strength Buffered Peptone Water (BPW).
17. Incubate flasks at 35oC +/-1oC for 20-24 hr.
18. Dilute inoculum at 10^{-2}, 10^{-3}, 10^{-4} and plate 50 µl of of samples over XLT4 agar.

Selective enrichment for *Salmonella*: in TT Broth and RV Broth

19. Transfer 0.5ml of samples enriched in non-selective broth to 10ml of TT broth.
20. Transfer 0.1ml of samples enriched in non-selective broth to 10ml of RV broth.
21. Incubate at 42°C for 22-24hrs.
22. Plate on XLT4 Agar. Duplicate plates per sample. Dilute to 10^{-4}, 10^{-5}, 10^{-6}.

23. Incubate at 35+/- 1°C for 22-24hrs.

24. Reincubate negative plates. Re-examine next day.

25. Examine for black or red colonies.

Reagents:

BB=Butterfield's Buffer: 0.5 mM Phosphate buffer, pH 7.2. [Ref. FDA Bacteriological Analytical Manual].

PBST= Phosphate Buffered Saline with Tween 80 or SPAN 80: 0.5 mM Phosphate buffer, 0.9% NaCl, 0.05% Tween 80.

INK= Carrageenan solutions (0.3%) are prepared aseptically by heating sterile water to 70°C. Then add the carrageenan by tapping the powder into the liquid. Break the lumps by using a sterile rod or homogenizer.

INK in BB= Carrageenan solutions (0.3%) are prepared aseptically by heating sterile BB to 70°C.

TT Broth= Tetrathionate [USDA FSIS Microbiology Guidebook.]

RV Broth= Rappaport Vassiliadis [USDA FSIS Microbiology Guidebook]

Detection of *Salmonella Spp.* from ground turkey extracted and isolated by inventive rinse agents

Trial 1	Ground turkey	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-		
1b	BB 2x	-	+	
2a	INK in BB 2x	-		
2b	INK in BB 2x	-	+	+
3a	INK-PBST-PBST	-		
3b	INK-PBST-PBST	-		

Trial 2	Ground turkey	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-	-	-
1b	BB 2x	-	-	-
			-	-
2a	INK in BB 2x	-	-	-
2b	INK in BB 2x	-	-	-
3a	INK-PBST-PBST	-	-	-
3b	INK-PBST-PBST	-	-	+

Trial 3	Ground turkey	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-	+	+
1b	BB 2x	-	+	+
2a	INK in BB 2x	-	+	+
2b	INK in BB 2x	-	+	+
3a	INK-PBST-PBST	-	+	+
3b	INK-PBST-PBST	-	+	+

Trial 4	Ground turkey	*Salmonella* spp.	*Salmonella* spp.	*Salmonella* spp.
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-	+	+
1b	BB 2x	-	+	+
2a	INK in BB 2x	-	+	
2b	INK in BB 2x	-	+	+
3a	INK-PBST-PBST	-	+	+
3b	INK-PBST-PBST	-	+	+

Results: These samples did not test positive for *Salmonella* after enrichment of rinse materials in non-selective broth. However, when enriched in selective broths (TT and RV) *Salmonella* spp. were detected in all test samples in Trials 3 and 4. In Trials 1, *Salmonella* was detected in 1b and 2b and in Trial 2, Sample 3b indicated positive. These results indicate that Salmonella were present in the ground turkey samples as injured cells and were revived in selective enrichment.

Modified Rinse Procedures. Samples 3 and 4 were precoated with 0.3% INK for 30 min at room temperature.

Trial 1	Ground turkey	*Salmonella spp.*	*Salmonella spp.*	*Salmonella spp.*
Sample No.	Treatment	Non-selective	TT Broth	RV Broth
1a	BB 2x	-	+	
1b	BB 2x	-	_	
2a	PBST 2x	-	+	
2b	PBST 2x	+	+	+
3a	INK-PBST-PBST	+	+	
3b	INK-PBST-PBST	+	+	+
4a	INK-ARG-PBST	+	+	
4b	INK-ARG-PBST	+	+	

The ground turkey samples in this modified procedure showed positive results for *Salmonella* spp. in samples 2, 3, and 4when treated with the inventive surface rinse materials and enriched in non-selected broth. These results indicated that *Salmonella* spp. proliferated in the non-selective culture. *Salmonella spp.* was not detected in Sample 1a and 1b when extracts were enriched in non-selective and RV enrichments broth. Blank date indicate no detectable *Salmonella*.

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Summary data on detachment of *E. coli* and coliforms from ground turkey and their detection with Coliform/*E. coli* Petrifilm test.

Sample No.	Treatment	Trial I Coliform	Trial I *E. coli*	Trial II Coliform	Trial II *E. coli*	Trial III Coliform	Trial III *E. coli*	Trial IV Coliform	Trial IV *E. coli*
1a	BB 2x	1			1			2	1
1b	BB 2x				1	2	1		
2a	INK in BB 2x		2		**5**		**2**	2	3
2b	INK in BB 2x				1, 1	3	1	2	**4**
3a	PBST 2x				1	**7**	**2**	**3**	
3b	PBST 2x		**3**		1, 1	1, 1	1	**3**	

Sample treatment, BB= Butterfield's buffer; PBST= phosphate buffer containing 0.9% sodium chloride and 0.05% Tween 80; INK = kappa-carrageenan. Each sample was plated at three dilutions, 10^0, 10^{-1}, and 10^{-2}. and applying 1 ml sample aliquots on Petrifilm. The manufacturer's guidelines and interpretation of results were followed. Results indicated by single numbers are detected at one dilution, typically in undiluted sample washes (10^0). When two numbers are reported, the bacteria are detected at 10^0 and 10^{-1}. The CFU reported indicate actual number per Petrifilm test (based on 1 ml sample) and are not adjusted to sample dilution. Samples without

numbers indicate that coliform or *E. coli* were not detected. The coliforms were indicated by red colonies with gas bubbles. Confirmed *E. coli* were indicated by blue to red colonies with gas bubbles.

These results indicate that the treated samples (# 2 and 3) showed enhanced detachment of *E. coli*. Blank data indicate no detectable coliform or *E. coli*. In trial I, *E. coli* was recovered in samples treated by the inventive rinse agents (#2 and #3). The original ground turkey sample in Trial III was stored for 8 days at 4°C and then treated with the inventive rinse agents to detach the bacteria followed by analysis with *E. coli*/Coliform Petrifilm. Coliform recovery was highest in samples treated in 2b, 3a, and 3b resulting in 81, 86 and 89 cfu/ml while 1a, 1b and 2a had 74, 24 and 61 cfu/ml. The numbers indicated in bold show the highest recovery of bacteria from the experimental treatment with inventive rinse materials.

Detachment of *Salmonella* spp, *E. coli/* and coliforms from whole chicken using the inventive rinse materials.

The following procedure is a modification of the FSIS procedure for rinsing a whole chicken (USDA/FSIS Microbiology Guidebook, 3rd Edition) and adapted to whole chicken legs (drumstick and thigh). The chicken was purchased from a local market.

Sample Preparation

1. Prepare four duplicate samples (#1,# 2, #3 and #4) of whole chicken legs (drumstick and thigh).
2. Drain packaging excess fluid from chicken part. Transfer chicken leg to sterile stomacher bags.
3. Coat samples #3 and #4 with 50 ml of 0.3% kappa carrageenan for 30 min at room temperature.
4. Squeeze fluid from chicken part and transfer coating fluid to a sterile 250 ml flask.
5. Rinse samples by adding 100 ml rinse solutions to the stomacher bags.
   #1. Butterfield's Buffer (BB)
   #2. Phosphate buffered saline with Tween 80 (PBST)

#3. Phosphate buffered saline with Tween 80 (PBST)

#4. 15% arginine-HCl

Rinse chicken with a rocking motion on a shaker at 4°C for 30 min.

6. Transfer and pool all rinse liquids from each sample into seaprate sterile 250 ml flasks. Store flask over ice to keep chilled.
7. Rinse the chicken samples again with

#1. Butterfield's Buffer (BB)

#2. Phosphate buffered saline with Tween 80 (PBST)

#3. Phosphate buffered saline with Tween 80 (PBST)

#4. Phosphate buffered saline with Tween 80 (PBST)

8. Adjust volume of rinse liquids to 250 ml with Butterfield's Buffer.
9. Make dilutions of 10^{0}, 10^{-1}, and 10^{-2}.
10. Test for coliform and E. coli using 3M Petrifilm by transfering 1 ml of diluted samples over the gel as directed by the manufacturer.
11. Incubate gel film for 24 hr at 37oC.
12. Examine results for gas producing red (coliform) and blue colonies (confirmed *E. coli*) and blue colonies without gas (presumptive *E.coli*).

Detachment of *Salmonella spp.* from whole chicken legs (drumstick and thigh) and their detection with XLT4 Agar.

Treat samples as in steps 1-8:

Non-selective enrichment for *Salmonella*:

13. Take out 30 ml aliquot of pooled liquid wash separately into four sterile 125 ml flask.
14. Add 30 ml of double strength Buffered Peptone Water (BPW).
15. Incubate flasks at 35oC +/-1°C for 20-24 hr.
16. Dilute inoculum at 10^{-2}, 10^{-3}, 10^{-4} and plate 50 μl of of samples over XLT4 agar.
17. Incubate at 35°C+/-1 for 22-24 hrs.
18. Examine for black colonies or yellow-red with black centers.

Salmonella spp. detection

Results: In four trials, black colonies were not observed in all tests, indicating that *Salmonella spp*. were not detected using the non-selective enrichment broth. [These experiments were carried out in the early phase of the project and sample rinses were not enriched in RV and TT selective broths.]

Reagents:

BB=Butterfield's Buffer: 0.5 mM Phosphate buffer, pH 7.2. [Ref. FDA/BAM]

PBST= Phosphate Buffered Saline with Tween 80 or SPAN 80: 0.5 mM Phosphate buffer, 0.9% NaCl, 0.05% Tween 80.

INK= Carrageenan solutions are prepared aseptically by heating sterile water to 70°C. Then add the carrageenan by tapping the powder into the liquid. Break the lumps by using a sterild rod or homogenizer.

Arg= 15% arginine-HCl

Summary data on enhanced detachment of *E. coli* and coliforms from whole chicken legs (drumstick and thigh) and their detection with Coliform/*E. coli* Petrifilm test.

Sample Number	Treatment	Trial I Coliform CFU/gel	Trial I Confirmed *E.coli* CFU/gel	Trial I Presump. *E.coli*	Trial II Coliform CFU/gel	Trial II *E.coli* CFU/gel	Trial II Presump. *E.coli*
1a	BB	8, 5, 1		29, 1			
1b	BB	1, 1		15, 1			1
2a	PBST			13		1	
2b	PBST	1		11			1
3a	INK+PBST	**60**		67, 7		1, 1	
3b	INK+PBST	1		**77**, 5, 3			
4a	INK+Arg+ PBST	1, 1		2, 1	1	1	
4b	INK+Arg+ PBST	1,1		2, 4			1

Sample Number	Treatment	Trial III Coliform CFU/gel	Trial III Confirmed *E.coli* CFU	Trial III Presump. *E.coli*	Trial IV Coliform CFU/gel	Trial IV Confirmed *E.coli* CFU/gel	Trial IV Presump *E.coli.*
1a	BB						
1b	BB					**1**	
2a	PBST		**6**				
2b	PBST	1		1			
3a	INK+PBST		3	**3,** 1	**1**		**2,** 1
3b	INK+PBST	**2**		2			1
4a	INK+Arg+ PBST						
4b	INK+Arg+ PBST		2				

Sample treatment, BB= Butterfield's buffer (FSIS sanctioned standard); PBST= phosphate buffer containing 0.9% sodium chloride and 0.05% Tween 80; INK = kappa-carrageenan; arg= arginine. Each sample was plated at three dilutions, 10^{0}, 10^{-1}, and 10^{-2}. One ml sample was applied on Petrifilm. The manufacturer's guidelines and interpretation of results were followed. Results indicated by single numbers are detected at one dilution, typically in undiluted sample washes (10^{0}). When 2 or 3 numbers are reported, the bacteria are detected at 10^{0} and 10^{-1}, and 10^{0}, 10^{-1}, and 10^{-2}, respectively. The CFU reported indicate actual number per Petrifilm test and are not

adjusted to sample dilution. Samples without numbers indicate that coliform or *E. coli* were not detected. Blank date indicate no detectable coliform or *E. coli*.

These results indicate that the treated samples (# 2, 3 and 4) showed enhanced detachment of *E. coli* and coliform. The coliforms were indicated by red colonies with gas bubbles. Confirmed *E. coli* were indicated by blue to red colonies with gas bubbles while the presumptive *E.coli* were indicated by blue colonies without gas bubbles. In trial I, the samples had higher number of bacteria but the sample #3a treated with carrageenan and PBST detached more bacteria than with the standard Butterfield's Buffer (BB). Results of Trial III at zero dilution is shown in Figure 9.

All of the references cited herein are incorporated by reference in their entirety, including the following:

U.S. Patent 6,172,040.

Medina, M. B., et al. (1997), Real-time analysis of antibody binding interactions with immobilized E. coli O157:H7, Biotechnology Techniques, Vol. 11 (3): 173-176.

Medina, M. B. (1997), SPR Biosensor: Food Science Applications, Food Testing and Analysis, Vol. 3 (5), 14-15, 36.

Medina, M.B. (1997), Studies on the attachment of *E. coli* O157:H7 on carcass components and inhibition/detachment with food additives, 1997 ARS/FSIS Research Planning Workshop, December, Riverdale, Maryland, Abstract.

Miller, A.J., et al. (1997), Microbial Safety Criteria for Foods Contacting Reuse Water in Food Processing Plants, 1997 Progress Report on Food Safety Research Conducted by ARS, ARS-USDA, Beltsville, MD, December 1997, pp. 80-82.

Medina, M.B. (1998), Mechanisms of Enterogenic Bacterial Attachment and Inhibition of *E. coli* O157:H7 Binding to Extracellular Matrix Proteins and Tissues, Presented at the 1998 Joint PAASE, DOST and UP Conference, Manila, Philippines (Abstract and presentation).

Medina, M.B. (1998), Biosensor Studies of Collagen and Laminin Binding with Immobilized *Escherichia coli* O157:H7 and Inhibition with Naturally Occurring Food Additives, Presented in SPIE's International Symposium on Industrial and Environmental Monitors and Biosensors: Pathogen Detection and Remediation for Safe Eating (EB20), Nov. 1-6, 1998, Boston, Massachusetts (Abstract).

Medina, M. B. (1998), Biosensor Studies of Collagen and Laminin Binding with Immobilized *Escherichia coli* O157:H7 and Inhibition with Naturally Occurring Food Additives, Proceedings of SPIE--The International Society for Optical Engineering, pp.97-104.

Medina, M.B., and P.F. Fratamico, P. F. (1998), Binding interactions of collagen I, laminin and fibronectin with immobilized E. coli 0157:H7 using surface plasmon resonance biosensor, Biotechnology Techniques, Vol. 12 (3): 235.

Palumbo, S.A., and M. B. Medina. (1998), Quantitative determination of pathogen reduction during animal slaughter and food processing to provide the scientific basis of HACCP and risk assessment,1998 Progress Report on Food Safety Research Conducted by ARS. ARS-USDA, Beltsville, MD, December 1998, pp. 90-100.

Medina, M. B., et al. (1999), Scanning Electron Microscopy Studies on Attachment of *Escherichia Coli* O157:H7 to Bovine Tissues, Presented at 1999 IFT Annual Meeting, Chicago, IL, July 24-28; Poster at Gordon Research Conference on Molecular Mechanisms of Microbial Adhesions (August 1-6, 1999) and at the 18th Annual Meeting of the Philippine-American Academy of Science and Engineering, Jekyll Is., GA, Aug. 13-15, 1999 (Abstract).

Median, M.B., et al. (2001), Inhibition of Escherichia coli O157:H7 attachment to collagen and bovine tissues by polysulfated polysaccharides, IFT Annual Meeting and Food Expo., New Orleans, LA, June 23-27, 2001 (Abstract available on-line, April 4, 2001).

Luchansky, J. B., et al.. (1999), Quantitative determination of pathogen reduction during slaughter and food processing to provide the scientific basis of HACCP and Risk Assessment, 1999 Progress Report on Food Safety Research Conducted by ARS, ARS-USDA, Beltsville, MD, December 1999, pp. 91-94.

Thus, in view of the above, the present invention concerns (in part) the following:

A method of detaching (completely or at least partially) microorganisms (e.g., bacteria) from, or of inhibiting (completely or at least partially) microbial attachment to, animal or poultry carcasses or seafood or parts thereof, said method comprising contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from, or inhibit microbial (e.g., bacterial) attachment to, said animal or poultry carcasses or seafood or parts thereof.

The above method, wherein said method is a method of detaching (completely or at least partially) bacteria from, or of inhibiting (completely or at least partially) bacterial attachment to, animal or poultry carcasses or seafood or parts thereof.

The above method, wherein said polysulfated polysaccharide is selected from the group consisting of heparan sulfate, dextran sulfate, lambda carrageenan, kappa carrageenan, iota carrageenan, and mixtures thereof.

The above method, wherein said polysulfated polysaccharide is heparan sulfate or dextran sulfate or lambda carrageenan or kappa carrageenan or iota carrageenan or mixtures of any of these.

The above method, wherein said polysulfated polysaccharide is kappa carrageenan.

The above method, said method comprising contacting animal or poultry carcasses or seafood or parts thereof at least twice with at least one of (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from, or inhibit microbial (e.g., bacterial) attachment to, said animal or poultry carcasses or seafood or parts thereof.

A method of detaching (completely or at least partially) microorganisms (e.g., bacteria) from animal or poultry carcasses or seafood or parts thereof, said method comprising contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of (i) a

polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) guanidine or arginine, optionally together with Tween and sodium chloride, or (iv) mixtures thereof, in an amount effective to detach microorganisms (e.g., bacteria) from said animal or poultry carcasses or seafood or parts thereof.

The above method, wherein said method is a method of detaching (completely or at least partially) bacteria from animal or poultry carcasses or seafood or parts thereof.

A method of inhibiting (completely or at least partially) microbial (e.g., bacterial) attachment to animal or poultry carcasses or seafood or parts thereof, said method comprising contacting animal or poultry carcasses or seafood or parts thereof at least once with at least one of (i) a polysulfated polysaccharide, or (ii) carboxymethyl cellulose, or (iii) mixtures thereof, in an amount effective to inhibit microbial (e.g., bacterial) attachment to said animal or poultry carcasses or seafood or parts thereof.

The above method, wherein said method is a method of inhibiting (completely or at least partially) bacterial attachment to animal or poultry carcasses or seafood or parts thereof.

Other embodiments of the invention will be apparent to those skilled in the art from a consideration of this specification or practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with the true scope and spirit of the invention being indicated by the following claims.